

## Repeated superovulation treatments in Kivircik ewes during breeding and nonbreeding seasons

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**Abstract:** The present study compared superovulation and embryo recovery rates applied repeatedly during breeding and nonbreeding seasons in the same 20 Kivircik sheep. Trials 1 and 3 were performed during the breeding season and the trial 2 was performed in the nonbreeding season. We synchronized the sheep's estrus cycles and induced a superovulatory response with porcine follicle stimulating hormone (pFSH) injections. Natural mating took place 12 h after the last FSH injection. The embryos were collected by laparotomy 7 days after natural mating. There was no significant difference between the number of corpora lutea (CL) counted following the 1st (breeding) and 2nd (nonbreeding) superovulatory treatments ( $P > 0.05$ ). However, the number of CL in the 3rd (breeding) treatment was dramatically lower than that in the other groups ( $P < 0.001$ ). Recovery rates showed significant differences among embryo flushing treatments ( $P < 0.001$ ). The number of morulae + blastocysts and freezable-quality embryos decreased considerably for the 3rd flushing treatment ( $P < 0.001$ ), while no significant difference was observed between the 1st and 2nd flushing treatments ( $P > 0.05$ ). In conclusion, our study demonstrated that 2 consecutive in vivo embryo production programs can be successfully applied in Kivircik ewes with suitable superovulation treatments and operation techniques, irrespective of the season.

**Key words:** Sheep, superovulation, embryo, FSH

### 1. Introduction

Turkey, which has a great number of endemic species, has an important share in the genetic resources of the earth (1). Kivircik, a sheep breed native to Turkey, has important social and economic value as genetic resources in livestock. Since 1991, the total number of sheep in Turkey has decreased dramatically. While in 1991 there were 39,000,000 head of sheep in Turkey, this number decreased to 25,000,000 in 2012 (2). Therefore, there is a need to develop strategies and methodologies to preserve and support the sustainability of native sheep breeds. Cryopreserved embryos might enable us to bring back sheep from a lost breed. Cryopreserving healthy germ cells and embryos may help to eradicate animal health problems and make use of specific genes in native breeds that might emerge in the future, as well (3,4). Developed countries have sought to preserve threatened breeds by offering financial incentives in the form of grants for the collection and cryopreservation of gametes and embryos (5).

Superovulation is the most important step in the conservation of animal genetic resources via in vivo

production. Multiple ovulation and embryo transfer (MOET) can be applied to gain extra genetic yield through the production of embryos obtained from selected females and males (6).

These programs in small ruminants are limited to the natural breeding season because small ruminants have seasonal cyclic activity patterns. In sheep, the breeding season lasts from August to November in temperate northern latitudes (7). Extending the application of these techniques outside of the breeding season would allow ewes involved in genetic improvement programs to undergo embryo recovery procedures throughout the year (8).

However, conflicting results have been reported in studies designed to determine the effect of the season in MOET protocols. Some studies of superovulation application in ewes from high latitudes have reported seasonal differences in the rates of ovulation (9), fertilization, and embryo quality (8). Such differences are not present or are smaller in tropical and Mediterranean areas (10).

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The variability in superovulation response can be attributed to extrinsic factors, such as the source and purity of gonadotropins and their application, and intrinsic factors, such as breed, age, nutrition, genetic variation, and ovarian status (11–15).

In addition to these factors, sheep embryos are surgically recovered, which often leads to the formation of postoperative adhesion in the reproductive tract, reducing the number of embryos collected after repeated surgeries (16); therefore, there is limited potential for repeated surgical flushings in the same animal (17,18). Research on the response of the Kivircik breed to ovarian stimulation, repeated uterus flushing, and evaluation of embryo quality during the breeding and nonbreeding seasons is limited (7).

The purpose of the present study was to compare superovulation and embryo recovery rates applied repeatedly during the breeding and nonbreeding seasons in the same Kivircik sheep.

## 2. Materials and methods

### 2.1. Animals and superovulatory treatments

The superovulation and embryo recovery applications were performed 3 times in 20 Kivircik ewes; 4 dead animals were removed from the final superovulation group. While trials 1 and 3 were performed during the breeding season (September–November), trial 2 was performed in the nonbreeding season (March–May). This study was carried out at the Uludağ University Applied Research Center for Veterinary Faculty in Bursa, located in northwest Turkey, at 40° north and 29° east and at an altitude of 120 m above sea level. During the trial, ewes were group-housed in straw-bedded pens with hay fed ad libitum and supplemented daily with 500 g of concentrate. All ewes were between 3 and 5 years of age with a mean body condition score of 3 (where 0 is extremely thin and 5 is obese) and a mean body weight of 50–60 kg.

To induce superovulation, an intravaginal sponge containing 45 mg of fluorogestone acetate (FGA) (Chronogest, Intervet, Turkey) was applied to each ewe on day 0. A total of 8 porcine follicle stimulating hormone (pFSH) (Folltropin 10 mL, 200 mg NIH-FSH-P1, Bioniche Animal Health, Ireland) intramuscular (im) injections were done twice a day (in the morning and evening) from day 12 to day 15, with doses of 1.5, 1.5, 1.5, 1.25, 1.25, 1.0, 1.0, and 1.0 mL. In addition, Prostaglandin $F_2\alpha$  (PGF $_2\alpha$ ; 250 µg cloprostenol, im Juramate, Jurox Pty Ltd, Australia) was applied to all ewes on day 12 and again in the morning of day 15, when the intravaginal sponges were removed. To stimulate ovulation, 1000 IU hCG (im, Chorulon, Intervet, Turkey) was applied to all the sheep on day 17. In order to avoid any variability in fertilization rate due to the intrauterine insemination technique, number of spermatozoa per dose, operator effect, fresh or frozen-

thawed semen, or other factors, only natural mating was used. Twelve hours after the last FSH injection, each ewe was placed for 48 h in a special mating cage with a ram of proven fertility.

### 2.2. Embryo recovery and assessment

The embryos were collected via mid-ventral laparotomy 7 days after natural mating. Feed and water were withheld from the ewes for at least 24 h prior to surgery. The ewes were anesthetized by im injections of 0.2 mg/kg atropine (atropine sulfate, Biofarma, Turkey) and 0.2 mg/kg xylazine (Alfazyne 2%, Alfasan International B.V., Holland), and intravenous (iv) injection of 22 mg/kg ketamine (Alfamine 10%, Alfasan International B.V., Woerden, Holland). Local anesthesia in the form of 2 mL of lidocane hydrochloride (Jetokain, Adeka Medical, Turkey) was also administered in the surgical area.

We assessed ovarian response by measuring the number of functional corpora lutea (CL) with good morphology. Uterine horns were exposed and flushed using a Foley catheter (12 FR) with embryo recovery medium (Lactate Ringer solution supplemented with 15% fetal calf serum) prewarmed to 38 °C. A stab incision at the base of the uterine horn close to the uterine bifurcation allowed for the insertion of a Foley catheter, which was then inflated with air until the tissue in contact with the ballooned area was taut. An open-ended 1/5 14-cm tom-cat catheter was introduced through a small puncture made at the utero-tubal junction in order to inject 20 mL of flushing medium into the uterine horn. This fluid was forced through the small opening in the Foley catheter at the base of the uterine horn and then collected into a sterile container; the process was repeated for the second uterine horn. The reproductive tract was flushed with a 2.5% heparin solution in saline before suture in order to minimize the postoperative development of abdominal adhesions. A general antibiotic was administered in the form of oxytetracycline (1 mL/10 kg body weight im Primamycin/LA, Pfizer, Turkey) and local antibiotic (Neo-Caf Spray, Intervet, Turkey) was applied at the site of the abdominal incision. After flushing, each donor was given a single injection of 250 µg PGF $2\alpha$  to prevent pregnancy from nonrecovered embryos. Despite surgical procedures being performed at different times, all were conducted by the same surgical team. Ethical concerns were always taken into account according to animal welfare regulations and practices. The flushed embryos were evaluated under a stereomicroscope (Nikon SMZ 1000) at a magnification of 20–60× and classified according to morphological criteria, using the guidelines of the International Embryo Transfer Society (19).

The embryos were classified as an unfertilized oocyte (UFO), 8–16-cell embryo, morulae + blastocyst, or transferable/freezable quality embryo. The total number of recovered and transferable/freezable embryos per ewe surgically flushed was recorded.

### 2.3. Statistical analysis

All data were analyzed with SPSS (version 20.0). The means of recovered unfertilized oocytes, 8–16-cell embryos, and morulae + blastocysts were calculated from individual donor ewes. The superovulation response was compared in the studied seasons by means of Friedman’s test. Results were expressed as mean ± SEM, and statistical significance was indicated by  $P < 0.001$ .

### 3. Results

Results for superovulatory responses and embryo yield values by repeated surgical embryo flushing operations are presented in the Table and Figure.

Following superovulatory treatments, there were no significant differences between the numbers of CL counted in the 1st (breeding) ( $8.8 \pm 1.3$ ) and 2nd (nonbreeding) ( $8.75 \pm 1.3$ ) superovulatory treatments ( $P > 0.05$ ). However, the number of CL in the 3rd (breeding) ( $3.8 \pm 0.8$ ) treatment was dramatically lower than that of the other groups ( $P < 0.001$ ).

Recovery rates were 68.75% (121/176), 58.85% (103/175), and 19.69% (13/66) for the 1st, 2nd, and 3rd treatments, respectively. Recovery rates showed significant differences among embryo flushing treatments ( $P < 0.001$ ).

In the 3rd flushing treatment, the mean number of morulae + blastocysts and freezable quality embryos ( $0.5 \pm 0.3$  and  $0.5 \pm 0.3$ ) was greatly reduced compared to the 1st ( $5.75 \pm 1.1$  and  $4.55 \pm 1.1$ ) and 2nd ( $4.0 \pm 0.9$  and  $3.4 \pm 0.8$ ) treatment groups ( $P < 0.001$ ), while no significant difference was observed between the 1st and 2nd flushing treatments ( $P > 0.05$ ).

There was no significant difference between the 1st, 2nd, and 3rd embryo flushing treatments in terms of the mean numbers of unfertilized oocytes ( $0.25 \pm 1.4$ ,  $1.05 \pm 0.7$ , and  $0.25 \pm 1.4$ , respectively ( $P > 0.05$ )).

### 4. Discussion

Since the cost-effectiveness of superovulatory treatments is a critical factor for the MOET techniques in small ruminants, the genetic value of the produced embryos should more than cover the expense of the superovulation, recovery, and cryopreservation procedures. The repeated use of selected ewes as donors could be a useful tool to reduce the unit cost of high-quality embryos.

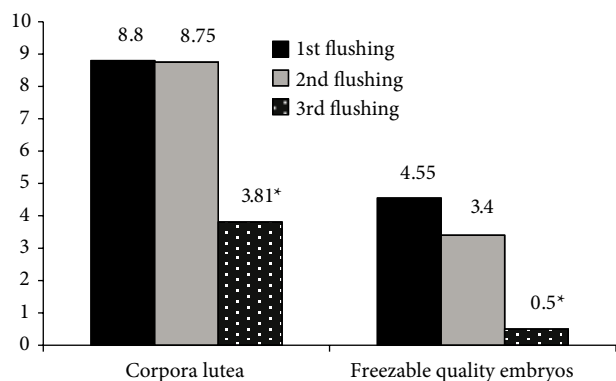
High variability in ovulation rate and the number of embryos recovered after superovulatory treatments in small ruminants can be attributed to the source and purity of gonadotropins and their application and to breed, age, nutrition, genetic variation, and ovarian status (11–15). Ovarian response was assessed by determining the number of CL. The mean numbers of CL obtained were  $8.80 \pm 1.3$ ,  $8.75 \pm 1.3$ , and  $3.81 \pm 0.8$  in the 1st, 2nd, and 3rd treatments of Kivircik ewes, respectively. The ovarian response after repeated superovulation tended to decrease with the 3rd FSH treatment, compared to the 1st or 2nd treatments, independent of the season in which the superovulatory protocol was performed ( $P < 0.001$ ). Similarly, Forcada et al. (20) observed a significant decrease in ovulation rate after the third FSH application in ewes. In goats, repeated superovulation with porcine FSH seems to reduce the ovulation rate after the third treatment due to the effect of anti-FSH antibodies, but the superovulatory response was maintained in this species treated several times (up to 5) with an ovine FSH preparation (21).

Aghdam et al. (7) and Torres and Sevellec (16) reported that repeated superovulation treatments had not affected ovarian response, although the results of our study contradict these data. In the present study the number of CL was affected by the repeated superovulation treatments. A difference in sheep breeds and source of gonadotropins might affect the ovarian response (7,22).

**Table.** Superovulatory treatment with decreasing doses of FSH response in Kivircik ewes in breeding and nonbreeding seasons.

	Superovulation treatment		
	Breeding season (1st treatment)	Nonbreeding season (2nd treatment)	Breeding season (3rd treatment)
Treated ewes	20	20	16
No. of corpora lutea (mean ± S.E.M.)	176 ( $8.80 \pm 1.3$ ) <sup>a</sup>	175 ( $8.75 \pm 1.3$ ) <sup>a</sup>	66 ( $3.81 \pm 0.8$ ) <sup>b</sup>
Recovery rate %	68.75 (121/176) <sup>a</sup>	58.85 (103/175) <sup>b</sup>	19.69 (13/66) <sup>c</sup>
No. of 8–16-cell embryos (mean ± S.E.M.)	1 ( $0.05 \pm 0.1$ ) <sup>a</sup>	2 ( $0.1 \pm 0.1$ ) <sup>a</sup>	1 ( $0.06 \pm 0.1$ ) <sup>a</sup>
No. of morulae + blastocysts (mean ± S.E.M.)	115 ( $5.75 \pm 1.1$ ) <sup>a</sup>	80 ( $4.0 \pm 0.9$ ) <sup>a</sup>	8 ( $0.5 \pm 0.3$ ) <sup>b</sup>
No. of unfertilized oocytes (mean ± S.E.M.)	5 ( $0.25 \pm 1.4$ ) <sup>a</sup>	21 ( $1.05 \pm 0.7$ ) <sup>a</sup>	4 ( $0.25 \pm 1.4$ ) <sup>a</sup>
No. of transferable/freezable quality embryo (mean ± S.E.M.)	91 ( $4.55 \pm 1.1$ ) <sup>a</sup>	68 ( $3.4 \pm 0.8$ ) <sup>a</sup>	8 ( $0.5 \pm 0.3$ ) <sup>b</sup>

a, b, c: Values with different superscripts in the same parameters are significantly different ( $P < 0.001$ ).



**Figure.** The mean number of CL and transferable/freezable quality embryos following superovulatory treatment.

\*Values with different superscripts in the same parameters are significantly different ( $P < 0.001$ ).

FSH promotes follicle growth but oocytes contained within these small follicles at the beginning of the treatment may lag behind in development (23). Follicles growing larger than 3 mm in size are able to develop a viable embryo (24). The nonbreeding season coincided with reduced ovarian activity in ewes and the percentage of small follicles in ovaries was greater than throughout the breeding season, even after FSH application (8). Thus, in the present study, an increased number of small follicles were induced to ovulate during the nonbreeding season compared to the breeding season, many of which contained immature oocytes, which cannot be fertilized, at the time of ovulation (8). The mean numbers of unfertilized oocytes were not significantly different among the flushing treatments ( $P > 0.05$ ). Repeated surgical recovery caused the development of adhesions in some ewes and thus hindered oocyte capture (16). In the present study, the increased number of unfertilized oocytes in the nonbreeding season treatment could be attributed to impaired sperm transport due to adhesions or to an increased number of immature oocytes. Although there were no significant differences among the 1st, 2nd, and 3rd treatment groups in terms of unfertilized oocytes, the number of unfertilized oocytes was highest according to recovered cell numbers (121 (4.1%), 103 (20.4%), and

13 (30.8%) for the 1st, 2nd, and 3rd treatment groups, respectively) in the 3rd group because of more severe adhesions.

Many studies have shown that using a surgical method to recover embryos results in a significant decrease in embryo recovery rates (16,25–27). Exteriorization of the reproductive tract often leads to the formation of postoperative adhesions of the uterus, oviducts, and ovaries to omental fat, thus inducing a reduction in embryo recovery after repeated surgery (16). Torres and Sevellec (16) reported that the formation of postoperative adhesions hardly impaired the percentage of embryo recovery or even sperm transport.

Although the use of heparinized saline solutions for flushing treatments can delay the development of such adhesions, recovery from genetically superior ewes often yields low numbers of embryos (16,20,28). In the present study, the season and the number of superovulation treatments had a significant effect on the ewe embryo recovery rate ( $P < 0.001$ ). However, comparing the 1st and 2nd superovulation treatments, the season did not negatively affect the mean number of morulae + blastocysts and freezable quality embryos in our study ( $P > 0.05$ ). The number of transferable embryos decreased significantly at the 3rd treatment ( $P < 0.001$ ). Similar results have been reported by Al-Kamali et al. (25) in ewes. This is in accordance with Ptak et al. (29), who found that follicular response declined with repeated stimulation. Therefore, the decrease in ovulations and transferable embryos after repeated treatments might be caused by genital tract adhesion after repeated flushings.

In conclusion, our study demonstrated that the efficiency of in vivo embryo production can be successfully applied in Kivircik ewes, irrespective of breeding and nonbreeding seasons, and that repeatedly administering a superovulation protocol did not impair in vivo embryo production until the 3rd superovulation treatment. These results could be used for other sheep breeds similar to Kivircik in terms of reproductive characteristics.

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